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## ORIGINAL ARTICLE

# Concentration of salivary protective proteins within the bound oral mucosal pellicle

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**OBJECTIVES:** To study which salivary proteins form the protective bound mucosal pellicle and to determine the role of transglutaminase in pellicle development.

**MATERIALS AND METHODS:** Oral epithelial cells were collected and underwent washes of different strengths, followed by homogenisation. SDS-PAGE, western blotting, IgA ELISAs and amylase activity assays were completed on cell homogenates and compared to saliva samples to confirm which salivary proteins were bound to cell surfaces.

**RESULTS:** Salivary mucins, MUC5B and MUC7, were strongly retained on the oral epithelial cell surface. Other bound proteins including cystatin S, carbonic anhydrase VI, secretory component and IgA could be washed off. IgA was present in concentrated levels in the bound mucosal pellicle compared to amounts in saliva. Amylase, one of the most abundant proteins present in saliva, showed minimal levels of binding. Transglutaminase 3 presence was confirmed, but proteins that it catalyses cross-links between, statherin and proline-rich proteins, showed minimal presence.

**CONCLUSION:** Some protective salivary proteins including mucins and IgA become concentrated on oral surfaces in the bound mucosal pellicle, through specific interactions. Concentration of mucins would contribute to lubrication to prevent abrasion damage to soft tissues, whilst increased IgA could create an 'immune reservoir' against mucosal infection.

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**Keywords:** saliva; pellicle; proteins; IgA; mucins

## Introduction

The formation of the saliva-derived protein pellicle on teeth is understood to provide protection and lubrication (Hannig *et al*, 2005). Less well known is the mucosal pellicle that acts as a physical barrier, controls the adhesion of unwanted pathogens and provides a lubricating layer on oral soft tissues (Bradway *et al*, 1989), essential for speech and mastication.

The formation of the acquired enamel pellicle in the mouth occurs within minutes after exposure of surfaces (Dickinson and Mann, 2006; Hannig and Hannig, 2009) and is reported to be between 30 and 100 nm (Lendenmann *et al*, 2000). On the teeth, statherin, proline-rich proteins (PRPs) and histatins are thought to initiate pellicle formation, followed by cross-linking and complexing of proteins to each other (Yao *et al*, 2000), by enzymes such as transglutaminase that form covalent bonds between  $\gamma$ -amide groups of a specific glutamine and an  $\epsilon$ -amino group of a specific lysine (Bradway *et al*, 1992; Yao *et al*, 1999; Hannig *et al*, 2008). This has been seen *in vitro*, where transglutaminase was able to catalyse a cross-link between acidic PRP-1 (24 kDa) and statherin (8 kDa) (Yao *et al*, 2000).

On the soft mucosal surfaces in the oral cavity, such as the buccal cells of the cheek, pellicle formation may also be enhanced by MUC1. This is a membrane-bound mucin (Cone, 2009), tethered on epithelial cells with an intracellular and extracellular domain (Gendler, 2001). It is a multifunctional protein, which not only might bind proteins involved in pellicle formation, but aid in protein and pellicle retention and may also play a role in signal transduction in the immune system function (Gendler, 2001). MUC1 has been shown to have a crucial role in mucus layer formation (Parmley and Gendler, 1998) and in the mouth is likely to form 'scaffolds' with gel-forming salivary mucin, MUC5B (Offner and Troxler, 2000).

Secreted salivary mucins, MUC5B and MUC7, are the most important glycoproteins found in saliva and the acquired enamel pellicle with regard to lubrication, due to their molecular properties (Tabak, 1995). MUC5B may make up part of the pellicle; it shows selective binding to

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hydroxyapatite, indicating interactions on the enamel surface, essential for the maintenance of the oral mucosa (Hannig *et al*, 2005; Cone, 2009). MUC5B has gel-forming properties and may physically form a protective barrier from pathogens (Raynal *et al*, 2003; Linden *et al*, 2008). Mucins also promote specific desired bacterial adherence to the oral tissues to aid the development of a commensal microflora, which colonise the oral cavity (Amerongen *et al*, 1995; Linden *et al*, 2008; Wickstrom and Svensater, 2008).

MUC7 could further aid in the immune functions of the pellicle by forming a heterotypic complex with sIgA and lactoferrin (Biesbrock *et al*, 1991; Soares *et al*, 2004). A similar synergistic property has been seen in the intestine with sIgA and mucins (Magnusson and Stjernstrom, 1982). This may aid in the development of an 'immune reservoir' in the salivary pellicle. IgA can promote the clearance of certain bacteria, which was also seen in the gut (Mantis *et al*, 2011), and is able to neutralise viruses within the oral cavity (McNabb and Tomasi, 1981). These protective immune properties of the pellicle are essential for health of the oral cavity. Other proteins such as statherin, agglutinins, PRPs, histatins and cystatins can aid in bacterial aggregation, preventing binding and colonisation in the oral cavity (Humphrey and Williamson, 2001).

The aim of this study was to investigate which (protective) proteins form part of the mucosal pellicle in the oral cavity because these may be very different to the enamel pellicle due to the nature of the surfaces. Lee *et al*, showed an increased concentration of salivary glycoproteins in residual saliva from the buccal mucosa compared to normally collected whole mouth saliva (WMS) (Lee *et al*, 2007), which may reflect the bound mucosal pellicle. A further aim was to determine whether there is presence of the cross-linking enzyme transglutaminase, could improve or alter binding of its substrates such as statherin and PRPs. For this reason, the present study examined pellicle proteins in oral epithelial cell homogenates with different strength washes to study protein retention on the cell surface.

## Methods

### Sample collection

Volunteers who gave informed consent, under ethical approval from NRES Committee London – Brent, were asked to provide oral epithelial cell samples by taking 10 ml tap water into the mouth and rubbing the cheeks against the molars for 1 min followed by expectoration into a universal tube (Satia *et al*, 2002). Samples were centrifuged at 170 *g* for 10 min, supernatant was removed and stored at  $-20^{\circ}\text{C}$ , and the cell pellet was re-suspended in 2 ml of ddH<sub>2</sub>O, where there is still a high number of cells that are not lysed in water (Michalczyk *et al*, 2004). Cell counts were then made using trypan blue to estimate live cell numbers. Two counts were made on a haemocytometer (Assistant, Germany) with an average number of live cells recorded and a total number estimated.

On a separate occasion, unstimulated WMS (UWMS) was also collected by drooling into a universal tube and parotid saliva (PS), stimulated by citrus sweets (Simpkins,

Sheffield, UK), was collected using a Lashley cup. Samples were divided into aliquots and frozen at  $-20^{\circ}\text{C}$ .

Comparisons with residual saliva and stimulated WMS (SWMS) were also desired. SWMS was collected for 5 min by chewing paraffin film. Residual saliva was collected using sialostrips placed and held on buccal surfaces for 10 s and then proteins were eluted by treatment with 20  $\mu\text{l}$  of 0.5 M DTT (1:10), LDS (1:4) and water and centrifuged at 657 *g* for 15 min in an Eppendorf tube.

### Oral epithelial cell washing

Oral epithelial cell samples were split into four 0.5-ml aliquots, each spun at 330 *g* for 3 min. The supernatant was then removed and stored for analysis and cell samples were then washed with 200  $\mu\text{l}$  of ddH<sub>2</sub>O, 50 mM Tris, 150 mM NaCl solution (TBS) or 0.2% sodium dodecyl sulphate (SDS). The control was left on ice for homogenisation. Cells were centrifuged at 330 *g* for 3 min; supernatants were removed and stored at  $-20^{\circ}\text{C}$ . All cell pellets were homogenised in 200  $\mu\text{l}$  of homogenisation buffer, 50 mM Tris containing 0.15% Triton X-100 at pH 7.3 with a protease inhibitor (1:100) (Calbiochem, Darmstadt, Germany) and stored at  $-20^{\circ}\text{C}$ .

### Protein analysis

Proteins remaining on the cells after washes were then compared to the control cells, which only underwent one water rinse, to determine which proteins were removed by which washes, indicating which proteins were more strongly bound to the oral epithelial cells. ImageJ (National Institute of Health, <http://rsb.info.nih.gov/ij>) was used to measure band intensity (Pramanik *et al*, 2010), following protein detection as described later, and area under the curves then compared. Cell protein levels were also compared to levels in saliva. An estimation of protein on soft tissue in the mouth was then based on the oral cell count calculated by Dawes (2003), 214.7  $\text{cm}^2$  ( $-20\%$  for enamel surface), and equivalent to approximately  $10^7$  cells (Dawes, 2003). Cell counts were used to estimate the amount of protein on the mouth surface, by expressing amounts of protein per  $10^7$  cells.

### Protein detection

SDS-PAGE was completed on cell homogenates, cell washes (data not shown) and saliva. Samples were added to LDS sample buffer (1:4) (Invitrogen, Paisley, UK) and 0.5 M DTT reducing buffer (1:10) (Invitrogen) and heated at  $100^{\circ}\text{C}$  for 3 min. 15  $\mu\text{l}$  of each sample was applied to each lane on a 4–12% Bis-Tris gel (Invitrogen). Electrophoresis was carried out in MES-SDS running buffer (Invitrogen) according to manufacturer's instructions. After completion of protein separation within the gel, staining with Coomassie Brilliant Blue R250 (CBB) (Sigma, Dorset, UK) was completed followed by destaining in 10% acetic acid.

### Periodic acid Schiff's stain (PAS)

Periodic acid Schiff stain was used to detect mucins following SDS-PAGE or following Coomassie staining post SDS-PAGE. Gels were fixed in 25% methanol and 10% glacial acetic acid for 1 h followed by a 20-min wash in

ddH<sub>2</sub>O. Oxidation in 2% periodic acid (Sigma) for 15 min was followed by a second and third wash in ddH<sub>2</sub>O for 2 min each. Schiff's reagent (VWR, Lutterworth, UK) was then added for 1 h in the dark under constant agitation, to complete the PAS.

#### Immunoblotting

Western blotting was completed according to the manufacturer's instructions and used to transfer proteins onto a nitrocellulose membrane. Immunoblotting was used to examine specific proteins of interest including MUC1 (1:1000) (a gift from John Hilkens, Netherlands Cancer Institute, The Netherlands), statherin (1:2000) (produced by Harlan Laboratories, Loughborough, UK, as described previously (Proctor *et al*, 2005)), cystatin S (1:2000) (R & D Systems, Abingdon, UK), carbonic anhydrase VI (CAVI) (1:1000) (R & D Systems), secretory component (1:500) (Dako, Ely, UK) and amylase (1:1000) (Sigma-Aldrich, Gillingham, UK). Antibodies against transglutaminases (TGM) 1 (Sigma-Aldrich), 2 (Abcam, Cambridge, UK) and 3 (Sigma-Aldrich) (all 1:2000) were also used to see whether TGM is present in the mucosal pellicle or saliva, and these were also compared to homogenised tissue-cultured TR146, HT29 and HT29-MTX cell lines, which we have found to express TGM 2 as a control. Membranes were blocked in either TBS with 1% Tween (TTBS) pH 7.6 or TTBS with 2% milk powder (Marvel) added. Membranes were probed with primary antibodies at room temperature for 1 h, washed in TTBS for 15 min and then followed incubation with the required secondary antibody. A final 15-min wash in TTBS was completed, and then, the membrane was incubated with a chemiluminescent substrate, 90 mM coumaric acid and 250 mM luminol with H<sub>2</sub>O<sub>2</sub> (Sigma). The membrane was then left to expose onto photographic film (Keyphoto, Harpenden, UK), developed and then fixed in the dark, followed by a water wash.

#### Enzyme-linked immunosorbent assay (ELISA)

IgA retention on all cell samples was measured using an ELISA, as previously used (Carpenter *et al*, 2000). Rabbit anti-human IgA 1:1000 (Dako, Ely, UK) in carbonate buffer was used to coat the ELISA plates overnight. Three washes in phosphate-buffered saline with 1% Tween (PBS-T) were completed. Samples were serially diluted down the plate in duplicate alongside the standard and incubated at 37°C for 1 h, followed by three more PBS-T washes. Detecting antibody rabbit anti-human IgA HRP 1:10000 was used for 1 h (Dako) followed by three final PBS-T washes. Substrate was then added consisting of 20 ml sodium acetate, with 3 µl of H<sub>2</sub>O<sub>2</sub> and 500 µl of 3, 3', 5, 5' tetramethylbenzidine (3 mg ml<sup>-1</sup> in dimethyl sulphoxide). The reaction was stopped with 2 M sulphuric acid, and absorbance was read at 450 nm using a microplate reader (BioRad, Hemel Hempstead, UK).

#### Amylase assay

An activity assay was used to determine the concentration of amylase in saliva and on cells. Samples of WMS (1:400 in amylase assay buffer) and oral epithelial cells were collected and compared to glucose standards

(0–4 mg) diluted in amylase assay buffer, 0.02 M NaH<sub>2</sub>PO<sub>4</sub>, 0.01 M NaCl, pH to 6.9 with NaOH, a modified method of the assay (Carmona *et al*, 1996). Fifty microlitre samples were heated for 3 min at 30°C, and then, an equal volume of 1% starch was added for 3 min. Finally, 100 µl of the colour reagent (1% dinitrosalicylic acid, 0.4 M NaOH with 30 g potassium sodium tartrate) was added, and samples were boiled for 5 min and then placed on ice. When all samples were ready, they were loaded in equal amounts onto a multiwell plate and absorbance was read at 540 nm using a microplate reader (BioRad).

#### Statistics

One-way analysis of variance (ANOVA) was used to compare mean amounts of protein on cell control and their decrease on cells following different types of washes. If significant, Student's *t*-test was carried out to compare between individual washes. *P* < 0.05 was considered significant after applying the Bonferroni's correction for multiple comparisons, where *P* < 0.016 was considered significant.

## Results

#### Proteins bound to oral epithelial cells

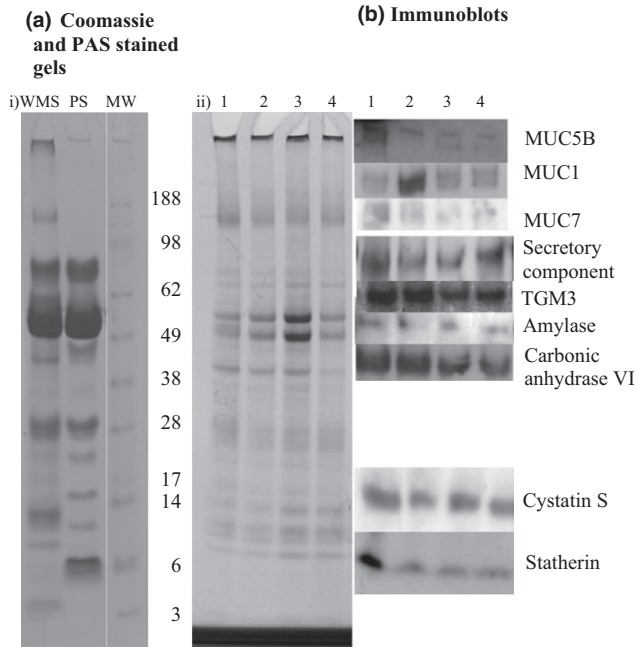
Most salivary proteins examined were found bound to the oral epithelial cells, as indicated by representative gels and western blots (see Figure 1). The mucins MUC5B and MUC7 were the most obvious salivary proteins detected by CBB/PAS staining of the oral epithelial cell homogenates (Figure 1a ii, lanes 1–4). Most other salivary proteins were visualised using immunoblotting techniques (see Figure 1b). This indicated the presence of several salivary proteins including CAVI, cystatin S, statherin and secretory component. Amylase was also present on immunoblots but at relatively lower levels compared to its abundance in saliva. PRPs, which are normally visualised as metachromatic (pink) staining using CBB R250, were not visible on the surface of the oral epithelial cells. Lastly, TGM 3 was found to be present in oral epithelial cell homogenates in both an active and inactive forms, whilst TGMs 1 and 2 were not detected (see Figure 2).

#### Elution of proteins from oral epithelial cells

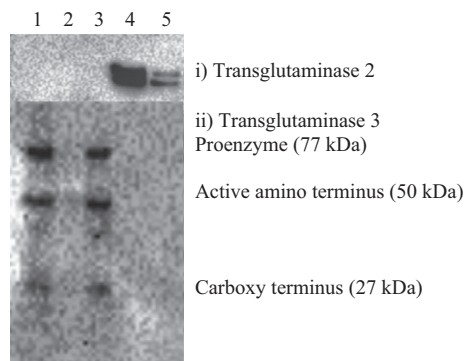
Oral epithelial cells were washed with ddH<sub>2</sub>O, TBS or SDS in order to elute proteins and to determine their protein binding strength to oral epithelial cells. Almost all proteins showed a degree of loss but this varied in its extent between different proteins (see Table 1). MUC1, an epithelial cell-bound protein, showed no loss from cells following water and TBS washes and an insignificant loss from SDS washes. This indicates that no cells were lost through the washes and protein retention levels should be reliable. The SDS concentration in the wash was previously adjusted to prevent cell lysis.

Of particular interest, both salivary mucins, MUC5B and MUC7, showed the highest levels of salivary protein retention after washes on the oral epithelial cells (see Table 1). The one-way ANOVA for both proteins showed





**Figure 1** SDS-PAGE gel electrophoresis and western blotting of saliva and oral epithelial cell homogenates following different washes. (a) Representative gel of four data sets, stained with CBB R250 and Periodic acid Schiff stain (PAS). Gel (i) unstimulated WMS (UWMS), PS and molecular weight markers in kDa (MW). In gel (ii), all lanes contain oral epithelial cell homogenates: control (1), ddH<sub>2</sub>O-washed (2), TBS-washed (3), SDS-washed (4). (b) Western blots probed with antibodies for different salivary proteins, representative of 3 or 4 volunteer samples, depending on the protein, see *n* number in Table 1



**Figure 2** Western blots of saliva and cell homogenates to determine the presence of transglutaminase in the oral cavity. Lanes: WMS (1), PS (2), oral epithelial cells (3), TR146 cells (4) and HT29 cells (5). (i) Blot for TGM 2 and (ii) blot for TGM 3 show both active and inactive forms. Western blots for TGM 1 showed no bands in saliva or any cells in the study

that there were no significant differences between the control and different wash groups for both proteins. On average, at least 66% or more of mucin was retained on the epithelial cell surface.

The ELISA quantitation of IgA in saliva and on cells (see Table 1) showed a significant (one-way ANOVA) decrease in protein on the surface of the epithelial cells following all wash types. After the Bonferroni correction, all washes removed a significant amount of IgA from the

epithelial cells, where  $P < 0.01$ . However, IgA still showed over 45% retention following all wash types. Secretory component showed similar retention levels as IgA. The one-way ANOVA showed a significant difference between the control and the other washed cell groups, where  $P < 0.01$ .

Statherin showed the lowest retention on the oral epithelial cells, and in one volunteer, statherin was not detected on the oral mucosal surface. In the TBS-washed cells, statherin reached levels as low as 30% of the amount found on the control cells. The one-way ANOVA showed a significant difference between the groups, where  $P < 0.01$ . TBS and SDS significantly reduced statherin levels compared to controls, where  $P < 0.01$  in both washes. Other salivary proteins cystatin S and CAVI both showed a significant decrease (one-way ANOVA) in retention on cells.

#### Protein amounts in saliva vs epithelial cell surfaces

The average concentration of IgA in parotid saliva was  $60.3 \pm 17.8 \mu\text{g ml}^{-1}$ . On the whole soft tissue surface of the mouth, there was estimated to be an average of  $206 \pm 38.9 \mu\text{g } 10^{-7} \text{ cells}$ . This was based on the amount of IgA on the control epithelial cells which numbered  $5.15 \times 10^5 \text{ cells ml}^{-1}$ . This was also much more than the known average amount of IgA in WMS.

#### Amylase activity on cells

In contrast to most other proteins, very little amylase adhered to oral epithelial cells. Immunoblots indicated low amounts of amylase on oral epithelial cells compared to the amount in 1 ml of saliva. The amylase assay indicated that there was virtually no enzymatic activity bound to epithelial cells (see Figure 3). Compared to that in saliva  $716.8 \mu\text{g ml}^{-1}$ , only  $4.3 \mu\text{g } 10^{-7} \text{ cells}$  of amylase were bound to oral epithelial cells.

#### Stimulated WMS, residual saliva and mucosal pellicle

Since such, a small amount of amylase was found bound to oral epithelial cells, a comparison of SWMS, mucosal saliva film and the epithelial cell pellicle was made, (Figure 4). SDS-PAGE analysis of samples was duplicated with one set of samples stained with CBB R250 and the other with PAS. This reconfirmed the minimal retention of salivary amylase on the oral epithelial cell pellicle compared to SWMS and residual saliva, and the concentration of IgA, an important immunoprotective protein.

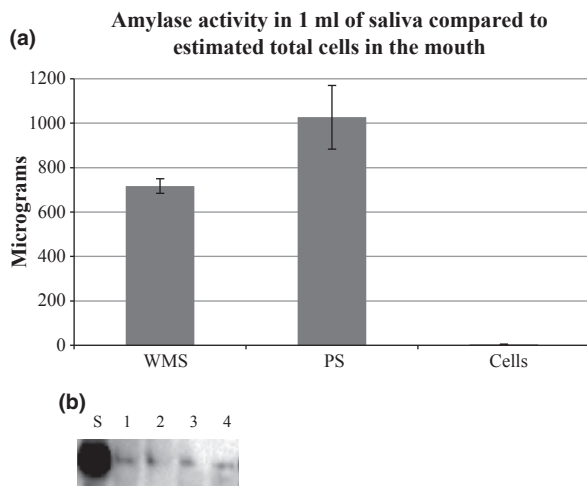
## Discussion

The relevance of the mucosal pellicle has been largely ignored and under investigated. Since Bradway *et al*, (Bradway *et al*, 1989, 1992) described its protein content, few papers have explored its role. In contrast, the benefits of the acquired enamel pellicle on teeth are well understood (Hannig *et al*, 2005). One potential reason for this might be the uncertainty as to whether the salivary proteins are bound or merely residual saliva. Two reports indicated considerable concentrations of proteins adhered to the oral mucosa in residual saliva (Lee *et al*, 2007;

**Table 1** Protein retention on oral epithelial cells after different washes: ddH<sub>2</sub>O, TBS and SDS

Proteins	n	Retention after water wash (%)	Retention after TBS wash (%)	Retention after 0.2% SDS wash (%)	Method of determination	ANOVA results of protein removal
MUC5B	4	71.2 ± 3.9	68.0 ± 5.0	73.6 ± 7.3	PAS	n.s.
MUC7	4	75.7 ± 13.6	66.2 ± 8.4	71.0 ± 12.4	PAS	n.s.
Cystatin S	4	70.3** ± 4.6	58.2 ± 15.0	38.7 ± 19.0	Western blot	<i>P</i> < 0.05
Secretory component	4	49.7* ± 5.9	41.6* ± 6.7	44.3 ± 15.0	Western blot	<i>P</i> < 0.05
IgA	6	62.5* ± 5.5	55.5** ± 4.9	46.0* ± 8.7	ELISA	<i>P</i> < 0.05
CAVI	4	83.8 ± 4.6	72.8 ± 10.9	42.3 ± 17.0	Western blot	<i>P</i> < 0.05
Statherin	3	49.7 ± 17.6	30.0* ± 6.3	35.8* ± 4.1	Western blot	<i>P</i> < 0.05
Amylase	3	69.6 ± 38.7	51 ± 44.0	53.2 ± 32.6	Western blot	<i>P</i> < 0.05
MUC1	3	116.8 ± 32.8	113.0 ± 22.3	81.6 ± 12.4	Western blot	n.s.

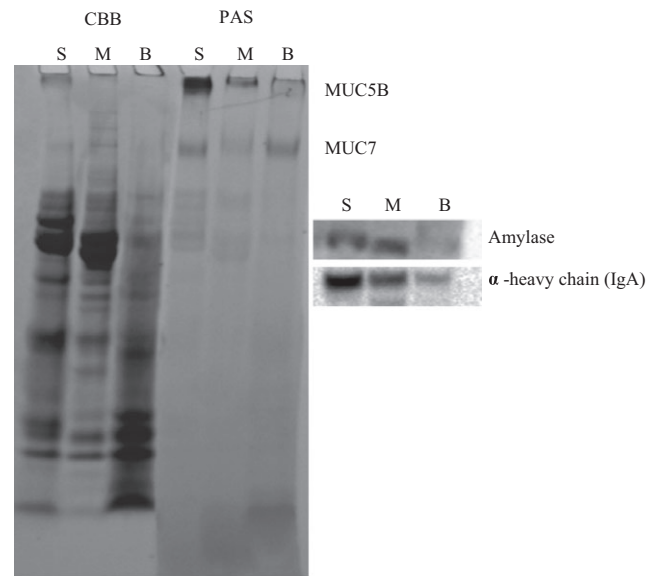
Results are expressed as a percentage of control, unwashed cells, ±standard error of the mean. One-way ANOVA indicated whether there was a difference in the washed cell groups from the control cells. \*Indicates the proteins significantly different from the control after a *t*-test, where *P* < 0.05 followed by the Bonferroni correction where *P* < 0.016. \*\*Indicates that the amount of protein was significantly different from control where *P* < 0.001.



**Figure 3** (a) Amylase assay activity of oral epithelial cells compared to WMS and PS. Graph displays amount of amylase activity in 1 ml of WMS and PS, and then, the amount of activity estimated on the oral epithelial cells (approximately  $10^7$  cells). (b) Immunoblot of anti-amylase on WMS (S), compared to amylase on cells (approximately  $10^4$  cells) control prewashed (1), water- (2), TBS- (3) and SDS-washed (4)

Pramanik *et al*, 2010). In this study, we have sought to confirm the avidity of salivary proteins bound to oral epithelial cells using different washes. Clearly, proteins that have resisted saline and detergent washes can be considered adherent and thus part of the mucosal pellicle. From our results in Table 1 and Figures 1 and 4, MUC5B, MUC7 and other proteins including sIgA should be considered as major components.

To further understand the relevance of the mucosal pellicle, we used the cellular-bound protein concentration to estimate how much total protein there would be in the whole mouth, using known cell counts and the estimate of approximately  $10^7$  soft tissue epithelial cells lining the mouth (Dawes, 2003). For proteins such as sIgA and mucins, the advantages of concentrating protein on the mucosa are increased lubrication (Slomiany *et al*, 1996; Inoue *et al*, 2008) and immune exclusion (McNabb and Tomasi, 1981). The specificity of the mucosal pellicle was well demonstrated by amylase.



**Figure 4** SDS-PAGE of S-WMS, M – mucosal saliva film and B – oral epithelial cells (approximately  $2.6 \times 10^6 \text{ ml}^{-1}$ ), showing bound pellicle. Gel on left stained with CBB R250, gel on right stained using Periodic acid Schiff stain (PAS). IgA, MUC5B and MUC7 show adherence to oral epithelial cells. Amylase shows almost no adherence in the bound mucosal pellicle

Amylase is the single most abundant protein in ductal saliva (Giometti and Anderson, 1980), and yet there was <0.6% of salivary amylase activity present on epithelial cells as seen in Figure 3. Table 1 and Figures 1 and 4 also indicated the lack of amylase present in comparison with other salivary proteins.

Likewise, few, if any, PRPs, which account for up to 70% of parotid salivary proteins (Beeley *et al*, 1991), were detected bound to oral epithelial cells. However, this could be due to the difficulties in visualising PRPs using western blotting techniques. Commercial antibodies are not available for PRPs, and instead, the metachromatic CBB R250 staining is used to visualise the proteins in gels. Lectin staining of basic PRPs (Proctor *et al*, 1997) revealed few, if any, PRPs (results not shown). However, this could be obscured by heavy staining of cell homogenates. Bradley

*et al* (1992) have shown indirect evidence of PRPs in the mucosal pellicle by tritium labelling, and Yao *et al*, (1999 and 2000) have shown the ability of PRPs, *in vitro*, to create cross-links with other salivary proteins such as statherin and histatins. In our study statherin was shown to be present, but in minimal amounts and western blotting was required to confirm presence. This could again suggest that any PRP present may be difficult to detect at low concentration levels. Lastly, it is possible that cross-linked proteins may form insoluble complexes, which may not resolve on the gel.

This study used three different washes to examine the nature of the pellicle–cell interaction. Water appeared to remove the least amount of protein; TBS more and SDS, a detergent commonly used as an elution solution for proteins from surfaces, removed most protein from cells compared to other washes. It is possible that a higher concentration, >0.2%, could have removed more proteins from the cells. The concentration used is approximately 0.82 times the critical micelle concentration (CMC) for SDS in water (Berg *et al*, 2001). However, it was found that higher SDS levels caused damage to the oral epithelial cells.

It is possible that there are a number of mechanisms responsible for the development of the mucosal pellicle. As mucin shows the highest retention, possibly due to a receptor on the oral buccal cells, this may initiate protein binding. However, many salivary proteins can bind to a large number of different surfaces. Lindh *et al* (2002) showed that statherin and acidic PRPs form films on a number of materials (Lindh *et al*, 2002). Hannig and Hannig (2009) have also proposed a model for pellicle formation that includes ionic and hydrophobic interactions alongside Van der Waals forces (Hannig and Hannig, 2009). This could ultimately lead to a protein-rich layer with heterotypic complexes. TGM 3 presence in oral epithelial cells in Figure 2 may also catalyse protein cross-links.

However, in another study with MUC5B, Lindh *et al*, also showed the importance of protein concentration in binding levels (Lindh *et al*, 2002). This may also indicate that all salivary proteins may bind to a different extent in all individuals due to their saliva composition. This is reflected in variations between protein binding on epithelial cells compared to the saliva concentration. However, it does suggest that there is a large amount of protein binding and it is likely that protein binding is highly influenced by the protein composition and concentration in an individual's saliva, resulting in very varied mucosal pellicles between different people. Amylase, however, did show very low levels compared to most other proteins in the oral pellicle, despite being the most abundant proteins in saliva, suggesting some type of specific interaction between proteins and the mucosa, ensuring it contains the protective proteins required for the maintenance of the oral soft tissues.

IgA has been found in the enamel pellicle (Lendenmann *et al*, 2000), residual saliva (Lee *et al*, 2007), and our results also confirm its presence in the mucosal pellicle at an increased concentration. IgA is secreted as sIgA into the oral cavity (Phalipon *et al*, 2002). Secretory component, also found present in the pellicle with similar

retention levels, is a polypeptide chain bound to IgA when in its secretory form. Its presence is thought to be essential to prevent colonisation by microorganisms (Phalipon *et al*, 2002). Phalipon *et al* (2002) principally showed that through its carbohydrate residues, secretory component aids in the adhesion of IgA to the mucosal surface whilst also protecting against proteolytic activity (Ma *et al*, 1998). It is also likely that some IgA is complexed with the well-retained MUC7, further aiding in its retention (Biesbrock *et al*, 1991). When amounts of IgA in the cell homogenates were used to estimate the amount of IgA in the bound mucosal pellicle, and compared to that in saliva, there was up to 5× as much IgA bound compared to 1 ml of parotid saliva and 2.5× values of IgA in WMS. Increased amounts of sIgA bound to the mucosa suggest that the pellicle is essential for immune protection, forming an 'immune reservoir' of protective proteins.

## Conclusion

Protective proteins such as MUC5B, MUC7 and IgA are present in concentrated levels creating a protective immune reservoir within the bound mucosal pellicle. The oral mucosal pellicle lacks PRPs and statherin, although TGM 3 is present and may aid in protein cross-linking, which suggests that formation of the pellicle is not driven by transglutaminase activity or statherin acting as a precursor. The formation of this pellicle may instead be initiated by salivary mucin binding to membrane-bound MUC1 (Cone, 2009) and by the development of a mucin 'scaffold' with MUC5B (Gendler, 2001) and further salivary proteins, especially sIgA.

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## Author contributions

Hannah Gibbins is the main author and carried out the experiments under the guidance of the other co-authors. Dr Carpenter, Prof Proctor contributed to the editing of the paper. They also aided with data interpretation. Both Mr Wilson and Dr Yakubov contributed to interpretation of the data. Experimental design was discussed among all authors and continually developed between myself and Dr Carpenter.

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## Supporting Information

Additional Supporting Information may be found in the online version of this article:

**Data S1.** Blots for data collected in Table 1.